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Ecophysiological and metabolic responses to interactive exposure to nutrients and copper excess in the brown macroalga *Cystoseira tamariscifolia*

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ABSTRACT

Global scenarios evidence that contamination due to anthropogenic activities occur at different spatial-temporal scales, being important stressors: eutrophication, due to increased nutrient inputs; and metal pollution, mostly derived from industrial activities. In this study, we investigated ecophysiological and metabolic responses to copper and nutrient excess in the brown macroalga *Cystoseira tamariscifolia*. Whole plants were incubated in an indoor system under control conditions, two levels of nominal copper (0.5 and 2.0 µM), and two levels of nutrient supply for two weeks. Maximal quantum yield (F_v/F_m) and maximal electron transport rate (ETR_{max}) increased under copper exposure. Photosynthetic pigments and phenolic compounds (PC) increased under the highest copper levels. The intra-cellular copper content increased under high copper exposure in both nutrient conditions. *C. tamariscifolia* from the Atlantic displayed efficient metal exclusion mechanisms, since most of the total copper accumulated by the cell was bound to the cell wall.

INTRODUCTION

Marine biota living in coastal waters are under constant threat from exposure to elevated concentrations of pollutants, such as metals and nutrients, mostly derived from domestic, industrial and farming activities (Ferreira et al. 2011). In near-shore ecosystems, macroalgae are the dominant primary producers; within the latter, brown seaweeds (Phaeophyceae) are particularly important bio-engineer organisms (Litter and Litter 1984, Wells et al. 2007), providing shelter, food and habitat for many other marine biota (Graham et al. 2007, Sáez et al. 2012).

Stress biology research on metal (and particularly copper)-stressed brown seaweeds has shown different levels of physiological, biochemical and molecular detrimental effects, as it has been observed in *Ascophyllum nodosum* (e.g. Connan and Stengel 2011a, 2011b), *Fucus vesiculosus* (e.g. Nielsen and Nielsen 2010) and *Ectocarpus siliculosus* (e.g. Roncarati et al. 2015, Sáez et al. 2015a). Even though copper is an essential metal at trace levels, for instance as co-factor in several enzyme complexes, beyond certain threshold concentrations it can become toxic and affect metabolic and physiological performance (Connan and Stengel 2011a, 2011b, Roncarati et al. 2015, Moenne et al. 2016). Copper excess can have negative effects on the metabolism of macroalgae through different known pathways (Sáez et al. 2015a). This involve the induction an oxidative stress condition and the substitution of other essential metals in biomolecules. In the case of the copper, this can replace magnesium in the chlorophyll molecule, incapacitating it to perform photosynthesis (Küpper et al. 2002, Moenne et al. 2016). In *A. nodosum* and *F. vesiculosus*, the ecophysiological responses were in detrimental under increase copper (1.6 µM for 15 d), causing an inhibition in photosynthesis and degradation of seaweed tips (Connan and Stengel 2011a, 2011b). In terms of metabolic responses, the copper at 2.4 µM for 7 d in the brown macroalga *E. siliculosus* showed increased levels of lipid peroxidation and H_2O_2 content with respect to without copper conditions, and displayed signs of oxidative stress and damage (Sáez et al. 2015a). Furthermore, *E. siliculosus* under increase copper at 2.4 µM increased antioxidant defences by means of increased content of phenolic compounds and greater production and activities of antioxidants and antioxidant enzymes, respectively, associated with the glutathione-ascorbate cycle were detected (Sáez et al. 2015a).

It is known that nitrate and phosphate represent important macronutrients for macroalgae development and in addition can protect the algae against stress. For instance, high concentrations of nutrients in seaweeds can reduce photoinhibition, as it has been observed in *Cystoseira tamariscifolia* under 50 µM nitrate (Celis-Plá et al. 2014a) and *Ulva lactuca* subject to 239 µM nitrate (Figueroa et al. 2009). Other observations showed that nutrient enrichment could also have positive effects on photosynthesis, photo-protection and biochemical responses (Celis-Plá et al. 2016). Indeed, *Cystoseira tamariscifolia* from Southern Mediterranean Sea showed that photosynthetic performance and the concentration of phenolic compounds were higher under 50 µM nitrate (Celis-Plá et al. 2014a). In contrast, individuals of *C. tamariscifolia* from ultraoligotrophic waters (Cabo de Gata-Nijar Natural park) showed greater photoinhibition and ecophysiological performance under 107 µM nitrate and 24 µM phosphate contents (Celis-Plá et al. 2014b). Certainly, the available information on the combined effects of metal-excess and increased nutrients is scarce in macroalgae; according to research available published, e.g., Huovinen et al. 2010. This study showed the most copper accumulation in *Macrocystis*, which decreased under nitrate-enriched conditions, as well as, the inhibition of photosynthetic activity by copper. Thus, the investigation on the combined effects of nutrients and metals excess studying in brown seaweeds would provide relevant information about their capacity to withstand the future pollution scenarios. The interaction between metals and nutrients excess is still not well understood for macroalgae. In this study, we analyse the physiological and biochemical responses under different copper and nutrient levels, using standard methods for the study of multiple physical stressors in algae (Martínez et al. 2012, Celis-Plá et al. 2014b). We investigate the interactive effects of excess copper and macronutrients (phosphate and nitrate) on certain parameters associated with physiological and metabolic responses in the brown seaweed *C. tamariscifolia*. Nitrogen and carbon internal content, photosynthetic pigments (chlorophylls and fucoxanthin), intracellular and released phenolic compounds, phenolic content, antioxidant capacity, and total and intra-cellular copper content, were measured. Additionally, photosynthetic activity was assessed by comparing parameters derived from measurements by using *in vivo* chlorophyll *a* fluorescence.

MATERIAL AND METHODS

Species, sampling and experimental design

Whole thalli of *Cystoseira tamariscifolia* (Hudson) Papenfuss, (Phaeophyceae, Fucales) (Gómez-Garreta et al. 2001, Bunker et al. 2010) were collected randomly on 6 May 2014 in Hannafore Point, Cornwall (50°36'N, 4°42'W), Atlantic Ocean. Seawater from this site have been described to have nitrate concentrations of around 5.0 μM (Woodward et al. 2013).

C. tamariscifolia (approximately 2 or 3 plants; in total 30 grs per open tank of the fresh weight of individuals) were incubated for 14 days (s), from 8 to 22 of May 2014 (after 48 hours of acclimation). The algal material was previously cleaned out of epiphytes manually under running seawater. The experiment was designed to examine interactive effects of copper (as $CuSO_4 \cdot 5H_2O$), at control copper (seawater with no copper added), at 0.5 μM (low copper levels) and 2.0 μM (high copper levels), and nutrient conditions, at control or natural seawater, and at 50 μM KNO_3 plus 5 μM KH_2PO_4 (nutrient enrichment). The six treatments were: control copper and natural seawater (CCNS); control copper and nutrient enrichment (CCNP+); low copper and natural seawater (LCNS); low copper and nutrient enrichment (LCNP+); high copper and natural seawater (HCNS); and high copper and nutrient enrichment (HCNP+). In total, 18 open tanks of methacrylate were used, with three replicates per treatment.

Experimental conditions

The experimental system consisted in 18 open tanks (0.030 m^2 surface area, 3.0 L volume), with seawater continuously aerated. Water temperature was monitored using a HOBO logger (Onset Computer Corporation, Massachusetts, USA). The photosynthetically active radiation PAR (λ=400-700 nm) was provided using cool white fluorescent lamps (Osram FH 21W/840HE, Luminox, Italy), and with a 14:10 h light/dark cycle. Seawater was changed every two days.

Physiological and biochemical variables

Several physiological variables were measured in the algae of each open tank after one week (7 days) and the end of the experiment (14 days). Nitrogen and carbon contents were determined in fronds using an element analyzer CNHS-932 model (LECO Corporation,

Michigan, USA) (according to Celis-Plá et al. 2016). Nitrogen and carbon were expressed as mg g $^{-1}$ dry weight (DW) after determining fresh weight (FW) to DW ratio in the tissue (8.17 for *C. tamariscifolia*).

Photosynthetic activity

In vivo chlorophyll *a* fluorescence associated with photosystem II was determined using a portable pulse amplitude modulated fluorometer Diving-PAM with a WinControl Software V3.25 (Walz GmbH, Germany). Pieces of the apical parts (one piece for replicate) of the fronds of *C. tamariscifolia* were collected at 7 days (middle time) and after 14 days (for each tank) and they were placed in the 10 ml incubation chambers in order to conduct rapid light curve, one for each tank. F_o (basal fluorescence yield) and F_m (maximum fluorescence yield) were determined after 15 min in darkness to obtain the maximum quantum efficiency of PSII (F_v/F_m), were $F_v=F_m-F_o$, F_o is the basal fluorescence of dark-adapted thalli after 15 min and F_m is the maximal fluorescence after a saturation light pulse of > 4000 µmol m^{-2} s^{-1}) (Schreiber et al. 1995). Electron transport rates (ETR, µmol electrons m^{-2} s^{-1}) as rapid light curve (RLC) was determined after 20 s exposure period in 12 increasing irradiance (9.3, 33.8, 76, 145, 217, 301, 452, 629, 947, 1403, 2084 and 3444 µmol m^{-2} s^{-1}) of white light, (halogen lamp of the Diving-PAM). ETR was calculated according to Schreiber et al. (1995) as follows:

$$ETR\ (\mu mol\ electrons\ m^{-2}\ s^{-1}) = \Delta F/F'_m \times E \times A \times F_{II} \quad (1)$$

Where $\Delta F/F'm$ is the effective quantum yield, $\Delta F = (Fm'-Ft)$, (Ft is the intrinsic fluorescence of alga incubated in light and F_m' is the maximal fluorescence reached after a saturation pulse of the algae incubated in light). E is the incident PAR irradiance expressed in µmol photons m^{-2} s^{-1}, A is the thallus absorptance as a fraction of incident irradiance that is absorbed by the alga (Figueroa et al. 2003), and F_{II} is the fraction of chlorophyll related to PSII (400-700 nm), being 0.8 in brown macroalgae (Grzymski et al. 1997). Maximum ETR (ETR_{max}, estimate of maximal photosynthetic capacity), and the photosynthetic efficiency (α_{ETR}) the initial slope of the ETR curve (estimate of photosynthetic efficiency) were obtained from the tangential function reported by Eilers and Peeters (1988).

Non-photochemical quenching (NPQ) was calculated according to Schreiber et al. (1995) as:

$$NPQ = (Fm - Fm')/Fm' \quad (2)$$

Maximal non-photochemical quenching (NPQ_{max}) is considerate as indicator of energy dissipation and as photoprotection mechanisms (Celis-Plá et al. 2016). NPQ_{max} was obtained from the tangential function of NPQ *versus* irradiance according to Eilers and Peeters (1988).

Pigment content

Pigments were extracted from 20 mg FW of fronds using 800 µL of dimethyl sulfoxide (DMSO) and 200 mL. After 5 min, samples were diluted with distilled water in a ratio of 4:1 (DMSO: water), and the absorbance (A) was determined at a spectrophotometer (Jenway 7315, Cole-Parmer, UK) at specific wavelengths (subscripts in equations below). Pigment concentrations are expressed as mg g^{-1} DW and calculated according to the following equations (according to Seely et al. 1972).

$$Chla = A_{665}/72.5 \quad (3)$$

$$Chlc = (A_{631} + A_{582} - 0.297\,A_{665})/61.8 \quad (4)$$

$$Fx = (A_{480} - 0.722(A_{631} + A_{582} - 0.297A_{665}) - 0.049\,A_{665})/130 \quad (5)$$

Total phenolic compounds

Total phenolic compounds (PC) were determined using 25 mg FW of fronds pulverized with mortar and pestle with sea-sand and 2.5 mL of 80% methanol. After storing the samples overnight at 4ºC, the mixture was centrifuged at 2253 g for 30 min at 4ºC, and the supernatant then collected. Total PC were determined colorimetrically using Folin-Ciocalteu reagent and phloroglucinol (1,3,5-trihydroxybenzene, Sigma P-3502) as standard. Finally, the absorbance was determined at 760 nm (Celis-Plá et al 2016). Total phenolic content was expressed as mg g^{-1} DW and the results are expressed as average ± SE of three independent replicates.

Phenolic compounds release

The phenolic compounds release (PR) in the seawater were determined by measuring the optical density at the maximal absorbance of polyphenols in the seawater, i.e., 270 nm (Celis-Plá et al. 2014a). The concentration, expressed as mg g^{-1} DW day^{-1}, was obtained using phloroglucinol dissolved in seawater as standard. PR was determined after 7 and 14 days of incubation.

Antioxidant capacity

The total antioxidant capacity was determined using the DPPH method (2,2-diphenyl-1-picrylhydrazyil) assay (i.e., AC) (Celis-Plá et al. 2016). The same extract for PC measurements was also used for DPPH analysis; 150 mL of DPPH were added to each extract. DPPH was prepared in 90% methanol (90MeOH: $10H_2O$) to a final concentration of 1.27 mM. The reaction was complete after 30 min incubation in the darkness at room temperature (~20°C). Absorbance was determined at 517 nm. DPPH concentrations (mM) were plotted against seaweed extract concentrations, expressed, as the AC, value (oxidation index; mg DW mL^{-1}) required for scavenging 50% of the DPPH in the reaction mixture. The calibration curve of DPPH concentrations was applied to calculate the concentration of DPPH remaining in the reaction mixture using ascorbic acid as control.

Single Phenolic Determination

The phenolic composition or single phenolic compounds (SP) were determined in the same extract as described for total phenolic compounds. The extract was filtered with 0.2 µm PVDF membrane filters. The SP were determined using ultra high-performance liquid chromatography (DIONEX UltiMate 3000 UHPLC, Thermo Scientific Inc.), equipped with a UV detector set at 260 nm (254 - 340 nm) (DIONEX MWD-3000, Thermo Scientific Inc.). The volume of injection was 20 µL per sample at 4ºC. PS composition was determined according to (Koivikko et al. 2007, Audibert et al. 2010) (according to commercial standards). Sixty polyphenols were found; shikimic acid (Sigma 69686), quinic acid (Sigma 46944-U), gallic acid (Sigma 91215), benzoic acid (Sigma 06185), quercetin (Sigma 1592409), kaempferol (Sigma 60010) and phloroglucinol (Sigma P-3502), with a retention time of 1.8, 2.4, 2.7, 17.03, 19.19, 20.57, 20.94 min, respectively. The chromatographic

separation was obtained using a C-18 reverse phase column (Supelco, Sigma-aldrich 15 cm x 2.1 mm, 3 μm) protected by a C18 guard cartridge (Security Guard, Phenomenex Inc., USA). The mobile phase consisted of two components: acetonitrile (solvent A); and 1% phosphoric acid in Milli-Q water (solvent B). PS were eluted using a gradient from 10% A for 2 minutes, 12% A for 3 minutes, 15% A for 1 minute, 30% A for 4 minutes, 35 % for 2 minutes, 50% A for 3 minutes, 35% A for 2 minutes, 3 minutes with 10% A and 90% B. Finally, an isocratic elution with 100% B was performed for the next 3 min.

Copper accumulation in C. tamariscifolia

After the experimental period and following removal of excess water, 40 mg fresh weight (FW) samples of algae were either immediately frozen at -80°C or washed twice for 15 min in Milli-Q water containing 10 mM EDTA to remove cell wall-bound copper (Roncarati et al., 2015). Thus, allowing distinction between total and intra-cellular (non-exchangeable) fractions (Hassler et al., 2004), and then frozen at -80°C. Frozen biomass were freeze-dried for 24 h and then digested with 2 mL of 70% (w/v) HNO_3 in a microwave oven (MARSX-press; cycle of 34 min at 120–170ºC). Digested samples were diluted to 5 mL with Milli-Q water and copper concentrations were determined by ICP-MS (Thermo Scientific, Hemel Hempstead, UK). External and internal calibrations of the instrument were achieved using copper certified standard solutions, and Itrium (193Ir) and Indium (115In), respectively. Certified reference material (*Fucus* spp. IAEA-140/TM) was treated in the same way as experimental material. Copper concentrations in reference material were 0.015 ± 0.003 μg g^{-1} DW.

Statistical analysis

Differences between physiological parameters in *C. tamariscifolia* were explored using a multivariable approach. A Principal Coordinates Analysis (PCO) was performed based on Euclidean distance using PERMANOVA + for PRIMER6 package. The overlay of the vectors onto the PCO was performed using Spearman correlation (Anderson, 2008). This procedure calculates the percentage variation explained by each of the axes in the multidimensional scale.

The interactive effects of the treatments on the physiological responses and biochemistry of *C. tamariscifolia* were assessed by ANOVA (Underwood, 1997). Three fixed factors were considered: time, with two levels (7 and 14 days); copper with three levels (CC, LC and HC); and nutrient enrichment with two levels (NS and NP+). This design allows testing for interactive effects of the ecophysiological variables (mean ± SE, n=3), with a level of probability at $p<0.05$ (Underwood, 1997). The Student Newman Keuls (SNK) *post hoc* test was performed if interactions were significant (Underwood, 1997). Homogeneity of variance was tested using the Cochran test and by visual inspection of the residuals. All data conformed to normality and homogeneity of variance. All analyses were performed using SPSS v.21 (IBM, USA).

RESULTS

Principal Coordinates Analysis

The principal coordinates analysis (PCO) (Fig. 1) shows that at 14d there was a positive correlation of the first axis (64.7% of total variation), with photosynthetic efficiency (α_{ETR}) and maximal non-photochemical quenching (NPQ_{max}), being highest in samples under CCNS treatments. In contrast, the maximal quantum yield (F_v/F_m), nitrogen internal content (N), chlorophylls *a* (Chl*a*) and *c* (Chl*c*), fucoxanthin (Fuco), intra-cellular (Cu_I) and total copper content (Cu_T), antioxidant capacity (AC), maximal electron transport rate (ETR_{max}), phenolic compounds (PC) and phenolic compounds in the seawater (PCw) were highest in samples collected at HCNS and HCNP+ treatments (Fig. 1).

Nitrogen (N) and carbon (C) internal content

Nitrogen had interactives effects between time x nutrient and copper x nutrient ($P<0.05$, Table S1). The only significant change was observed at the end of experiments (14d). The N was lower with respect to the increased copper with nutrient enrichment and non-enrichment treatments (Fig. 2a), but at the middle of the experimental period, the N was higher respect to initial experimental time. Carbon had no significant differences (Table S1). Nevertheless, the C has a trend increase under high copper with nutrient and non-nutrient enrichment (Fig. 2b).

Photosynthetic variables

The maximum quantum yield (F_v/F_m) was significantly affected by the interaction between copper x nutrient (P<0.05, Table S2). F_v/F_m increased significantly under high copper with nutrient enrichment treatment during the experimental period (Fig. 3a). The photosynthetic efficiency (α_{ETR}), had a significantly interaction among all factors (P<0.01, Table S2). The α_{ETR} was highest under high copper with non-nutrient enrichment treatments, at the middle the experimental period (Fig 3b); in addition, the α_{ETR} was higher during the experimental period respect to the initial values. The maximal electron transport rate (ETR_{max}), had significant interactions between time x copper and copper x nutrient (P<0.05, Table S2). ETR_{max} increased in high copper with non-nutrient enrichment conditions during the experimental period (Fig. 4a); in addition, the ETR_{max} in all treatments were lower than the beginning of the experimental period. Finally, the maximal non-photochemical quenching (NPQ_{max}) presented interactive effects among all factors (P<0.01, Table S2). The NPQ_{max} was highest under control copper with nutrient enrichment treatments during the experimental period, as well as, in lower copper with non-nutrient enrichment at middle the experimental time (Fig. 4b).

Photosynthetic pigments

Chlorophyll *a* (Chl*a*), Chlorophyll *c* (Chl*c*), and fucoxanthin (Fuco) contents had significant differences between time x copper (P<0.05, Table S3). All pigments increased under high copper nutrient enrichment and non-nutrient enrichment treatments at the end of experimental period (Fig. 5a, b and c).

Phenolic compounds and antioxidant capacity

The phenolic compounds (PC) had significant differences among all factors (P<0.01, Table S3). PC increased under higher copper with nutrient and non-nutrient enrichment treatments, in low copper with non-nutrient and in control copper with nutrient enrichment treatments, at the end the experimental period (Fig. 6a). The phenolic compounds release in the seawater (PR) had interactive effects among all factors (P<0.05, Table S4). The PR were higher at middle the experimental time under high copper with nutrient enrichment treatments (Fig 6b). The antioxidant capacity (AC) presented differences significant among all factors

342 (P<0.05, Table S4). In the middle and at the end of experimental period, the AC was higher
343 under high copper with nutrient enrichment and non-enrichment nutrient treatments (Fig. 6c);
344 in addition, the AC was higher respect to the initial experimental period (Fig. 6c).
345 Phenolic compounds through UHPLC were detected in all treatments, as shikimic acid and
346 phloroglucinol. However, quinin acid, gallic acid, benzoic acid, quercetin and kaempferol
347 were observed only as traces (Table 1). Shikimic acid showed significant differences
348 (P<0.05, Table S5) for the time factor, and phloroglucinol showed significant differences
349 between time x nutrient (P<0.05, Table S5). Shikimic acid increased under high copper with
350 nutrient enrichment treatments, and phloroglucinol compound was higher under low and high
351 copper with nutrient enrichment conditions. Both compounds were higher compared to levels
352 at the beginning of the experimental period (Table 1).
353
354 ***Copper accumulation***
355 Total copper content (intra-cellular plus extra-cellular) increased significantly upon copper
356 exposure (P<0.01, Table S6). After experiments, the maximal total accumulation was found
357 around 260-μg g^{-1} DW under high copper with nutrient and non-nutrient enrichment (Fig.
358 7). Similarly, the intra-cellular copper content in *C. tamariscifolia* increased in parallel upon
359 levels of copper exposure. Accumulation was not significantly influenced by nutrient
360 enrichment. In spite of the level of nutrient inputs, intracellular copper concentrations were
361 always about half of the total accumulation (Fig. 7).
362
363 **DISCUSSION**
364 Recent reviews surmise that ecophysiological responses as photosynthetic performance and
365 metabolism can be negatively affected by exposure to excess copper in brown seaweeds,
366 although these studies have not considered eventual modified effects mediated by
367 macronutrient availability (Connan and Stengel 2011a, 2011b, Ryan et al. 2012, Sáez et al.
368 2015a). At ecological point of view, this study shows as nutrient availability influences the
369 effects of copper on the algal metabolism. Thus, in a scenario of eutrophication, the negative
370 effects of copper of algal physiology could be reduced. In terms of nutrient enrichment in *C.*
371 *tamariscifolia*, Figueroa et al. (2014) and Celis-Plá et al. (2014a) have demonstrated that high
372 nutrient levels of up to 50 μM KNO_3 plus 5 μM KH_2PO_4 enhance photosynthesis,

photoprotection, the production of photosynthetic pigments and antioxidant capacity in *C. tamariscifolia* from the Mediterranean Sea. In contrast, in this investigation, *C. tamariscifolia* from the Atlantic showed no clear differences in photosynthetic, photoprotective and antioxidant effects under exposure of up to 50 µM KNO_3 plus 5 µM KH_2P_4. In this regard, it is important to mention that average nutrient concentrations in seawater nearby the collection site of *C. tamariscifolia* used for this study, can be found around 2.25-5.0 µM of nitrate and 0.32-1.0 µM of phosphate (Woodward et al. 2013), whereas seawater in the Mediterranean, an oligotrophic Sea where *C. tamariscifolia* was collected, has nutrient levels of approximately 1.59 µM of nitrate and 0.15 µM of phosphate (Ramírez et al. 2005). It is possible then, that *C. tamariscifolia* from the Atlantic has sufficient baseline intracellular nutrient concentrations for metabolic processes. It is important to consider that most nitrogen and phosphorus are incorporated in inorganic and organic forms through active transport. In this context, it has been observed that nutrient availability is an important mediator of the expression of genes encoding nitrogen and phosphorus membrane transporters; these are having been observed to be down regulated under nutrients excess in other Heterokonts (Wurch et al. 2014). The information suggests that *C. tamariscifolia* from the Atlantic, under high nutrient levels and given its sufficient intracellular nutrients, is employing active transport mechanisms to avoid excess nitrogen and phosphorus in the inner cell, not affecting trends in photosynthesis, photoprotection, production of photosynthetic pigments and antioxidant capacity mediated by copper excess (see below).

In vivo chlorophyll fluorescence parameters (maximal quantum yield, F_v/F_m, as indicator of photoinhibition, increased mainly at the 2.0 µM of copper exposure and nutrient levels, under both 7 and 14 d experiments. In contrast, Connan and Stengel, (2011b) found no changes in F_v/F_m in *Ascophyllum nodosum* and *Fucus serratus* exposed to 0, 1.6 and 24 µM copper for 15 d. This suggest that the interaction with nutrient levels maybe can help to increase at the maximal quantum yield in *C. tamariscifolia* this study, we shown that the maximal electron transport rate (ETR_{max}) or photosynthetic production increased under low copper and natural seawater, in both experimental times. Connan and Stengel (2011b) shown that under high copper treatments (1.6 - 24 µM of copper), the rETR was 100 - 120 µmol e^- m^{-2} s^{-1} for *A. nodosum*, and 90 - 100 µmol e- m^{-2} s^{-1} for *F. vesiculosus*, whereas the controls presented 220 and 250 µmol e^- m^{-2} s^{-1}, respectively. This suggests that the interaction of the copper with

nutrient enrichment reduced the photoinhibition in the PSII. Huovinen et al. (2010) observed inhibition of photosynthetic activity, i.e., low F_v/F_m, under copper for 12 d, in three brown algae, *Durvillaea antartica*, *Macrocystis pyrifera* and *Lessonia nigrescens*, with the strongest response in the latter species. These authors demonstrated that the nitrate enrichment mitigated the inhibitory effect of copper on photosynthesis in all three species. Interestingly, mechanisms of energy of dissipation (NPQ_{max}) decreased with increasing nutrient levels and under high copper exposure. In our investigation, general trends show that there was a decrease in NPQ_{max} with increasing copper exposure, at both 7 and 14 d, despite level of nutrients. Connan and Stengel, (2011b) shown that under high copper treatments (24 µM), the NPQ_{max} increased with respect to control conditions for *A. nodosum*; in contrast, in *F. serratus* under 1.6 - 24 µM copper, NPQ_{max} did not demonstrate differences if compared to controls. In our study, the NPQ was lower took place, under high copper with natural seawater and high copper with nutrient enrichment in the seawater, when the presence of the photosynthetic pigments was higher, this suggest that was related to the xanthophyll cycle, and photoprotection mechanisms (Celis-Plá et al. 2014a).

The chlorophylls *a* (Chl*a*) and *c* (Chl*c*), and fucoxanthin increased mainly under high copper treatments under both nutrient levels. Nielsen and Nielsen, (2010) showed that in *F. serratus* under 0.84 µM copper with high irradiance, pigment contents were 2.0 mg g^{-1} Chl*a*, 0.50 mg g^{-1} Chl*c* and 0.15 mg g^{-1} fucoxanthin. In our study, we found similar values in 2.0 µM of copper concentrations for Chl*a*, Chl*c* and fucoxanthin. This is in agreement with the results gathered by Sáez et al. (2015a), which observed increasing fucoxanthin production upon greater copper exposure of up to 2.4 µM in a copper-tolerant strain of *E. siliculosus*. Fucoxanthin is the main xanthophyll and light harvesting pigment in brown seaweeds; moreover, it has been found that fucoxanthin *in vitro* has strong antioxidant capacity (Sachindra et al. 2007, Mikami et al. 2013). Despite the latter, no evidence on the role of fucoxanthin as an antioxidant within the metabolism of brown macroalgae has been proved. As it is known that copper excess induces an oxidative stress condition in seaweeds, is then possible that fucoxanthin is acting as an antioxidant to avoid or, at least, diminish metal-mediated oxidative damage in the chloroplast. The latter is relevant taking into account that excess of copper-induced reactive oxygen species (ROS) is importantly produced through the disruption of electron transport chains in the chloroplast (Moenne et al. 2016).

435 Despite the treatment with no copper addition and nutrient enrichment, there was no clear
436 influence of nutrient excess on total phenolic compounds (PC). Indeed, this is in agreement
437 with investigations on different macroalgae species under nutrients excess, which displayed
438 no changes in PC content (Pfister and Van Alstyne 2003, Van Alstyne and Pelletreau 2000).
439 Concerning copper stress, PC increased mainly after 14d culture, and subjected principally
440 to high copper. This information is also in according with several investigations that show
441 that copper excess mediates greater PC content in brown macroalgae due to their strong metal
442 chelating and antioxidant capacities (Sáez et al. 2015a, Costa et al. 2016). Respect to the
443 single phenolic compounds, the results showed the induction of shikimic acid, gallic acid and
444 phloroglucinol; furthermore, and especially after 14d culture, it was observed an additive
445 effect of nutrients and copper excess in the production of shikimic acid and phloroglucinol.
446 In regard to nutrients, it has been shown that rice plants watered regularly with up to 357 µM
447 nitrogen for 7 d displayed increased expression of genes associated with the phenylalanine
448 metabolism, responsible for the synthesis of phenolic compounds (Xiong et al. 2010). In
449 relation to copper excess, phlorotannins induction has been described for brown macroalgae,
450 and respond to their active role as metal chelators and ROS scavengers (Connan and Stengel
451 2011b, Sáez et al. 2015a, Moenne et al. 2016).
452 The decrease of the internal phenolic compounds or intracellular compounds, respect to of
453 the phenolic compounds release or extracellular compounds, in macroalgae, may be related
454 to a greater release to the outer media in order to fulfil a photoprotective function and to
455 provide a barrier to avoid excess-radiation mediated-stress (Celis-Plá et al. 2014a, 2016).
456 Thus, phenolic compounds in the extracellular media or release (PR) were higher under high
457 copper levels with control of seawater and enrichment nutrient seawater. This information is
458 interesting since it has been described that brown algae release metal-complexing substances
459 (including phenolic) during copper excess, to diminish extracellular bioavailable
460 concentrations and avoid excess copper entering the cell (Gledhill et al. 1999). Although the
461 latter has not been observed in brown macroalgae under nutrients excess, it may be possible
462 that nutrient-mediated induction of PC intracellularly is causing a release of non-required PC
463 to the extracellular media. Total antioxidant capacity in *C. tamariscifolia* was enhanced under
464 high copper with natural seawater, but it decreased at high copper and nutrient enrichment,
465 at both experimental times. While there does not seem to be an influence of nutrient excess

on antioxidant responses, there is an enhanced antioxidant capacity induced by increased copper concentrations, in agreement with published data. Indeed, the information may imply that higher antioxidant capacity induced by copper excess in *C. tamariscifolia* is caused by the activation of the glutathione-ascorbate cycle, the most important antioxidant mechanism in photoautotrophs, as it has been described in *E. siliculosus* under metal excess in laboratory and field transplantation experiments (Sáez et al. 2015a and 2015b).

Concerning copper accumulation, the results show that intracellular, extracellular and total accumulation in *C. tamariscifolia* increase upon levels of copper exposure, despite excess nutrients. It has been postulated that the cell walls in brown macroalgae have an important role in cellular exclusion mechanisms, constituting a first barrier to avoid metal excess intracellularly during periods of high concentrations in the external media (Moenne et al. 2016). For instance, it is known that alginic acid and sulphated polysaccharides in brown algae cell walls provide strong binding sites for the chelation of bioavailable metals (Davis et al. 2003). In this investigation, it was observed that extracellular accumulation was in general half of total accumulation in all experimental treatments, which is in agreement with copper exclusion patterns observed in species as *E. siliculosus* (Roncarati et al. 2015) and *Lessonia berteroana* (Andrade et al. 2006). Thus, *C. tamariscifolia* of Atlantic waters display efficient copper exclusion mechanisms that prevent metal excess intracellularly through extracellular copper accumulation. High nutrient level (nitrate and phosphate) has a positive effect against the toxical effect by Cupper.

CONCLUSIONS

In this study, we demonstrated that *C. tamariscifolia* from the Atlantic, display differential responses to nutrients and copper excess. The nutrient excess did not induce an increase in photosynthetic performance, as observed in other studies on *C. tamariscifolia* from the Mediterranean Sea, suggesting that intra-specific differences were mostly induced by dissimilarities in baseline intracellular nutrient concentrations mediated by environmental levels from where the algae were collected. Pigments content (specifically fucoxanthin) were greater at the highest copper exposure, which may indicate a contribution as an antioxidant in the chloroplast. Moreover, while intracellular phenolic compounds responded mainly to copper excess, polyphenols release seemed to be importantly mediated by both high nutrients

and copper excess, although only at 7 d of experiments. Interestingly, the photosynthetic responses, although not major, responded principally to copper excess. In addition, photosynthetic and light harvesting pigments increased mainly by induction of copper excess. Copper exclusion mechanisms appeared to be efficient in *C. tamariscifolia*, since extracellular accumulation was generally half of total copper accumulation, despite external nutrients and levels of copper exposure. Important aspects that arise for future investigations in *C. tamariscifolia* are the inter-population differences in nutrient absorption and influence in metal stress metabolism, in addition to the potential role of fucoxanthin to counteract oxidative stress in the chloroplast. The biomass of Atlantic *Cystoseira tamariscifolia*, with high polyphenol content under high cupper and high nutrient levels, could have both ecological and cosmeceutical implications. This algal biomass could be useful for the extraction of polyphenols for cosmeceutical use due to they have a great number of beneficial effects associated to their cosmetic and pharmacological properties. In addition, at ecological level, brown algae in copper polluted and eutrophic waters can contribute to the bioremediation of natural waters.

Acknowledgements

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Table 1 Phenolic composition, Shikimic acid, Quinic acid, Gallic acid, Benzoic acid, Quercetin, Kaempferol and Phloroglucinol (mg g^{-1} DW) (mean values ± SE, n=3) of *Cystoseira tamariscifolia.* In relation to final od experimental period, control copper x natural seawater (CCNS), control copper x nutrient enrichment (CCNP+), low copper x natural seawater (LCNS), low copper x nutrient enrichment (LCNP+), high copper x natural seawater (HCNS) and high copper x nutrient enrichment (HCNP+). Initial time of the experimental period is shown in the first column. Lower-case letters denote significant differences after SNK test after 7 days and capital letters denote significant differences after SNK test after 14 days. *nd*: not detected.

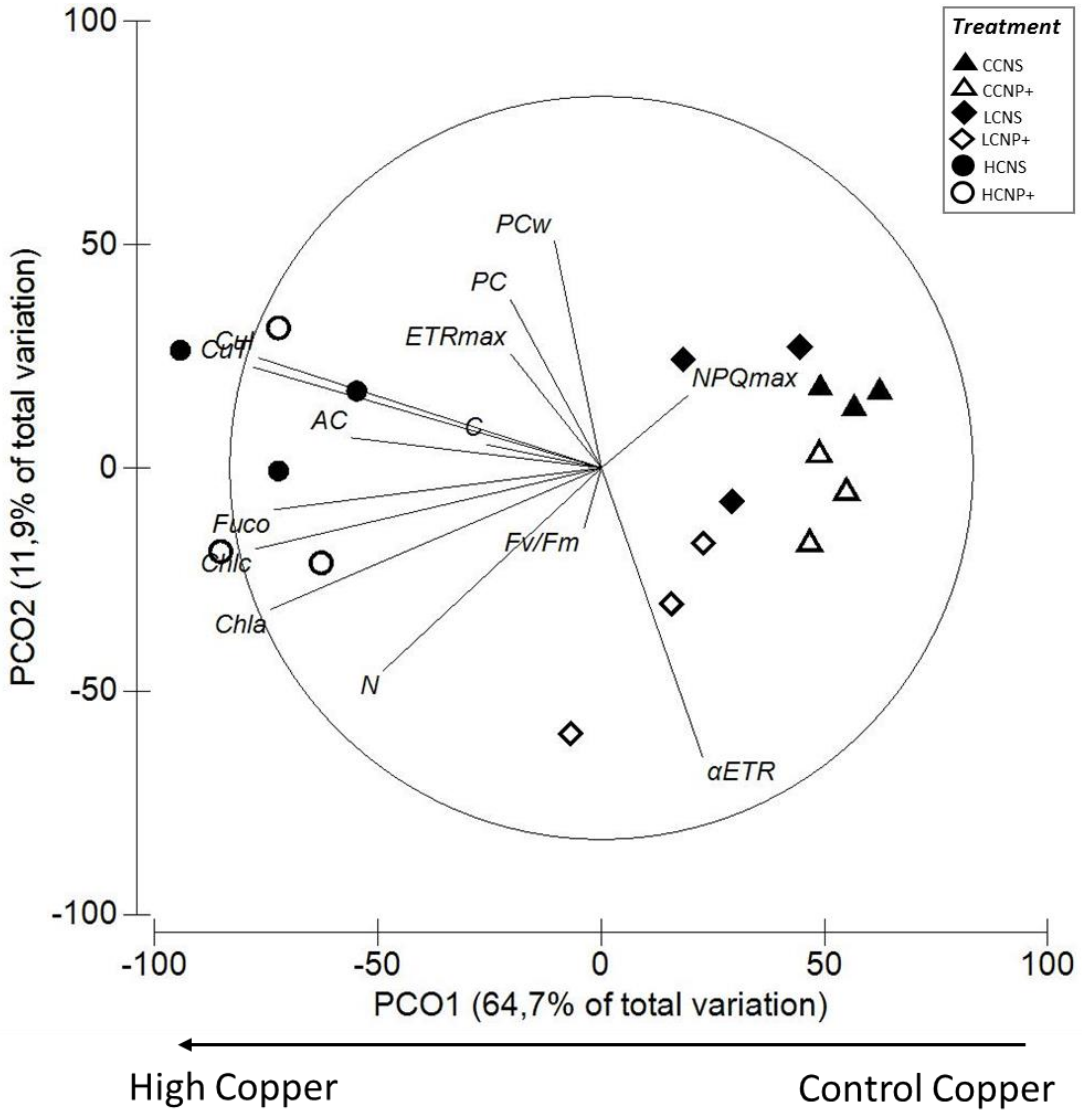
		Phenolic composition						
	Treatments	***Shikimic acid***	***Quinic acid***	***Gallic acid***	***Benzoic acid***	***Quercetin***	***kaempferol***	***Phloroglucinol***
	It	5.67 ± 0.32	0.06 ± 0.01	0.32 ± 0.08	0.06 ± 0.02	1.46 ± 0.01	0.26 ± 0.01	0.91 ± 0.36
After 7 days	*CCNS*	1.67 ± 0.38^{b}	*nd*	0.21 ± 0.01^{a}	*nd*	*nd*	*nd*	0.76 ± 0.05^{a}
	CCNP+	1.72 ± 0.07^{b}	*nd*	0.16 ± 0.02^{a}	*nd*	1.42 ± 0.01	*nd*	1.99 ± 0.02^{a}
	LCNS	0.54 ± 0.09^{a}	*nd*	0.14 ± 0.01^{a}	*nd*	0.17 ± 0.02	*nd*	2.92 ± 0.03^{b}
	LCNP+	1.73 ± 0.13^{b}	*nd*	0.18 ± 0.01^{a}	0.82 ± 0.01	*nd*	*nd*	3.53 ± 0.23^{b}
	HCNS	1.76 ± 0.11^{b}	*nd*	0.16 ± 0.01^{a}	*nd*	*nd*	*nd*	5.51 ± 0.51^{c}
	HCNP+	2.12 ± 0.07^{b}	*nd*	0.42 ± 0.01^{b}	*nd*	*nd*	1.47 ± 0.04	6.94 ± 0.63^{c}
After 14 days	*CCNS*	2.39 ± 0.18^{A}	*nd*	0.15 ± 0.01	1.31 ± 0.01	*nd*	*nd*	2.39 ± 0.18^{A}
	CCNP+	2.33 ± 0.06^{A}	*nd*	0.16 ± 0.01	*nd*	*nd*	*nd*	3.81 ± 0.45^{AB}
	LCNS	2.75 ± 0.05^{A}	*nd*	*nd*	*nd*	*nd*	*nd*	4.48 ± 0.02^{B}
	LCNP+	3.01 ± 0.01^{A}	*nd*	0.57 ± 0.01	*nd*	*nd*	*nd*	13.12 ± 1.01^{D}
	HCNS	5.48 ± 0.44^{B}	*nd*	*nd*	*nd*	*nd*	*nd*	5.48 ± 0.03^{A}
	HCNP+	9.46 ± 0.68^{C}	*nd*	0.56 ± 0.01	*nd*	*nd*	*nd*	9.91 ± 0.42^{C}

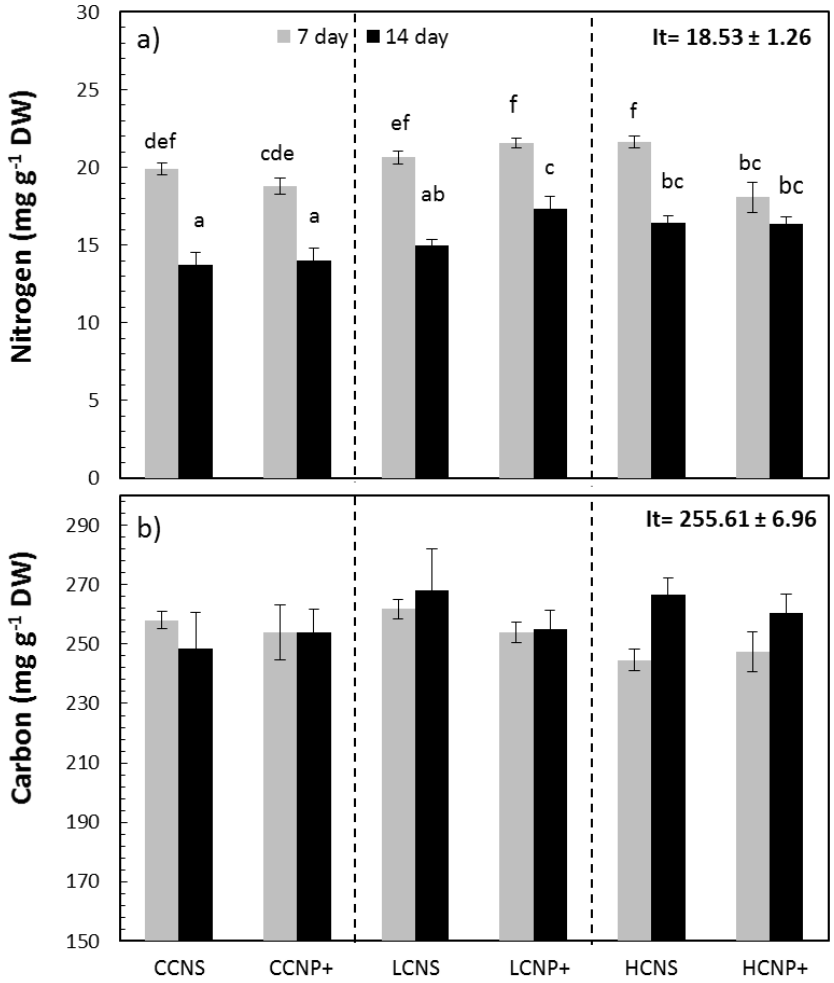
691 **FIGURE CAPTIONS**
692
693 Figure 1 Principal component analysis (PCO) of *Cystoseira tamariscifolia* after experimental
694 period respect to variables. Maximal non-photochemical quenching (NPQ_{max}),
695 photosynthetic efficiency (α_{ETR}), phenolic compounds in the seawater (PCw), phenolic
696 compounds (PC), maximal electron transport rate (ETR_{max}), maximal quantum yield (F_v/F_m),
697 carbon (C) and nitrogen (N) internal contents, intra-cellular (Cu_I) and total copper content
698 (Cu_T), antioxidant capacity (AC), fucoxanthin (Fuco), chlorophylls *a* (Chl*a*) and *c* (Chl*c*). In
699 relation to final od experimental period, control copper x natural seawater (CCNS), control
700 copper x nutrient enrichment (CCNP+), low copper x natural seawater (LCNS), low copper
701 x nutrient enrichment (LCNP+), high copper x natural seawater (HCNS) and high copper x
702 nutrient enrichment (HCNP+).
703
704 Figure 2 Nitrogen and carbon internal content of *Cystoseira tamariscifolia.* In relation to
705 time (7d, grey bars and 14d, black bars), CCNS, CCNP+, LCNS, LCNP+, HCNS and
706 HCNP+ treatments. Upper values in right box indicate initial time, values represent means ±
707 SE (n=3) and lower-case letters denote significant differences (p<0.05) after a SNK test.
708
709 Figure 3 Effect of copper on nutrient of photosynthetic parameters; a) F_v/F_m (maximal
710 quantum yield) and b) α_{ETR} (photosynthetic efficiency) of *Cystoseira tamariscifolia.* In
711 relation to time (7d, grey bars and 14d, black bars), CCNS, CCNP+, LCNS, LCNP+, HCNS
712 and HCNP+ treatments. Upper values in right box indicate initial time, values represent
713 means ± SE (n=3) and lower-case letters denote significant differences (p<0.05) after a SNK
714 test.
715
716 Figure 4 Effect of copper and nutrient of photosynthetic parameters; a) ETR_{max} (maximal
717 electron transport rate) and NPQ_{max} (maximal non-photochemical quenching) of *Cystoseira*
718 *tamariscifolia.* In relation to time (7d, grey bars and 14d, black bars), CCNS, CCNP+,
719 LCNS, LCNP+, HCNS and HCNP+ treatments. Upper values in right box indicate initial
720 time, values represent means ± SE, n=3 and lower-case letters denote significant differences
721 (p<0.05) after a SNK test.

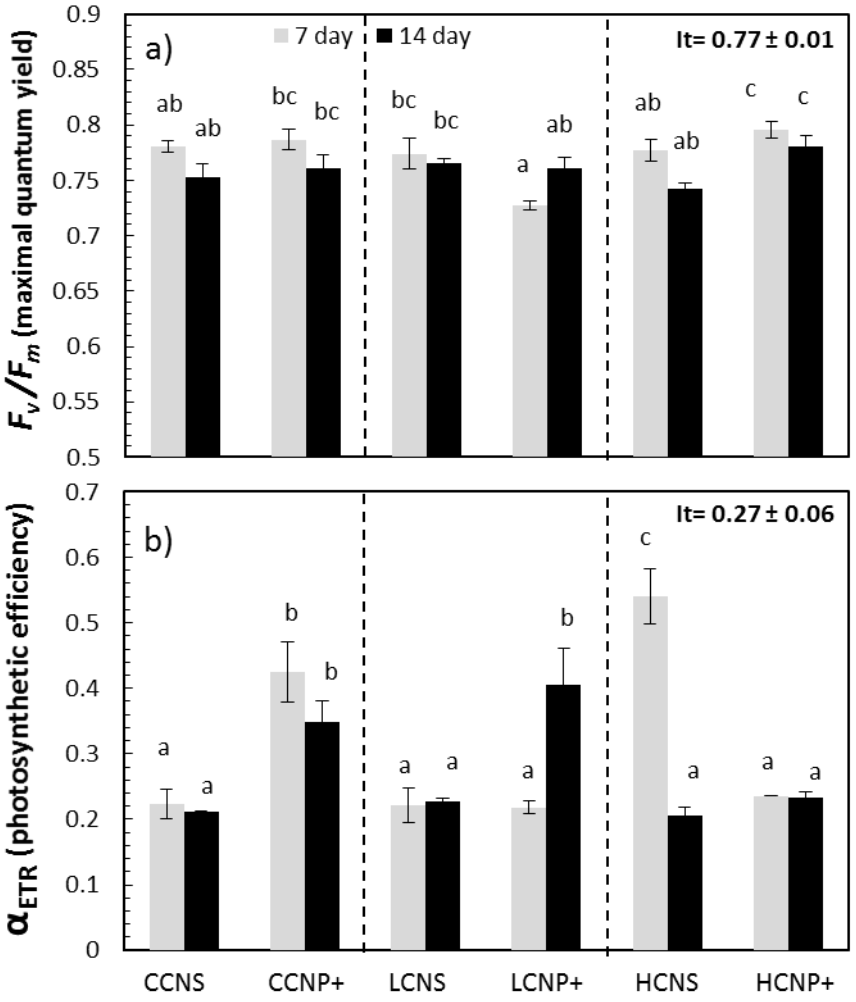
Figure 5 Photosynthetic pigment a) Chl*a* (Chlorophyll *a*), b) Chl*c* (Chlorophyll *c*) and c) fucoxanthin (express as mg g^{-1} DW) of *Cystoseira tamariscifolia*. In relation to time (7d, grey bars and 14d, black bars), CCNS, CCNP+, LCNS, LCNP+, HCNS and HCNP+ treatments. Upper values in right box indicate initial time, values represent means ± SE (n=3) and lower-case letters denote significant differences (p<0.05) after a SNK test.

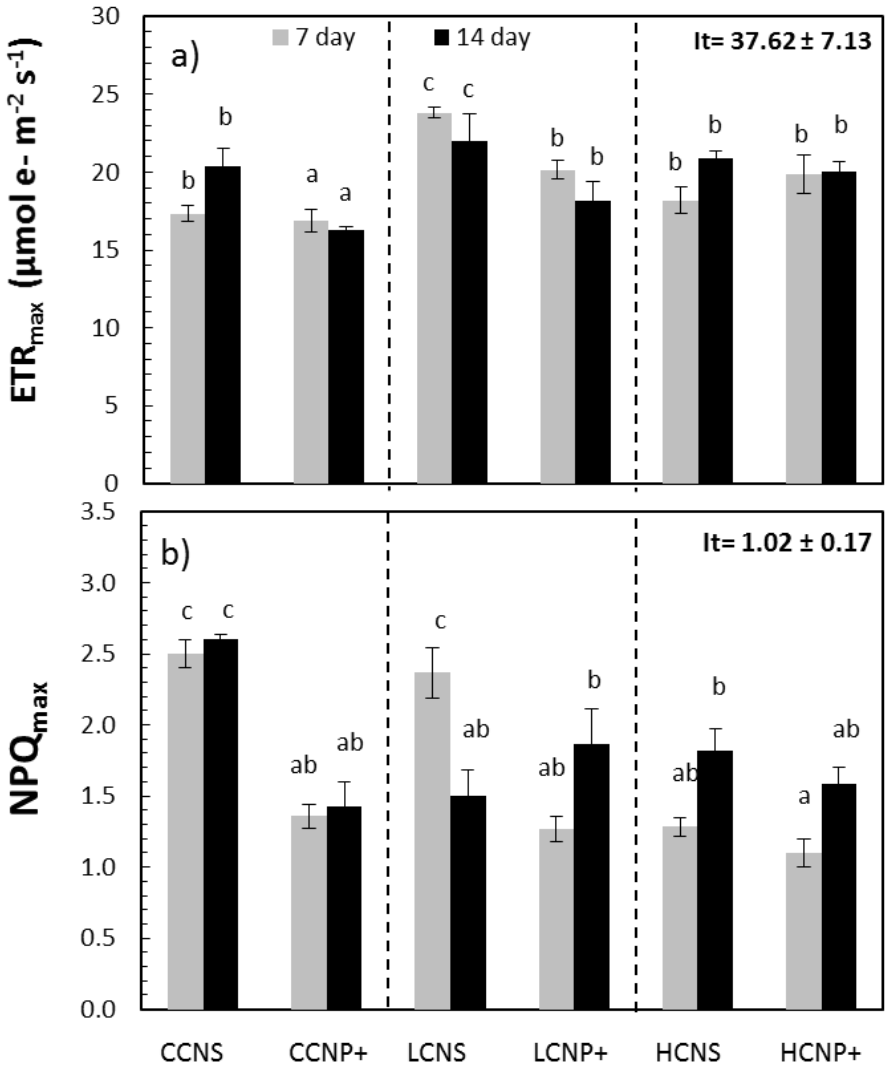
Figure 6 Photoprotective compounds; a) Phenolic compounds (mg g^{-1} DW), b) PCw (phenolic released) (mg g^{-1} DW d^{-1}) and c) antioxidant capacity (express as $EC_{50,}$ mg DW mL^{-1}) of *Cystoseira tamariscifolia*. In relation to time (7d, grey bars and 14d, black bars), CCNS, CCNP+, LCNS, LCNP+, HCNS and HCNP+ treatments. Upper values in right box indicate initial time, values represent means ± SE (n=3) and lower-case letters denote significant differences (p<0.05) after a SNK test.

Figure 7 Intra-cellular (grey bars) and total copper concentration (black bars) after experimental period, in relation to CCNS, CCNP+, LCNS, LCNP+, HCNS and HCNP+ treatments. Capital letters denote significant differences (p<0.05) in intra-cellular copper concentrations, and lower case letters denote significant differences in total copper concentration in *Cystoseira tamariscifolia* after SNK test.


Treatment
CCNS
CCNP+
LCNS
LCNP+
HCNS
HCNP+
PCw
PC
ETRmax
Cutl
AC
C
NPQmax
Fuco
Chlc
Fv/Fm
Chla
N
αETR
100
50
0
-50
-100
-100
-50
0
50
100
PCO2 (11,9% of total variation)
PCO1 (64,7% of total variation)
High Copper
Control Copper


a)
7 day
14 day
It= 18.53 ± 1.26
Nitrogen (mg g-1 DW)
30
25
20
15
10
5
0
def
a
cde
a
ef
ab
f
c
f
bc
bc
bc
b)
It= 255.61 ± 6.96
Carbon (mg g-1 DW)
290
270
250
230
210
190
170
150
CCNS
CCNP+
LCNS
LCNP+
HCNS
HCNP+


a)
7 day
14 day
It= 0.77 ± 0.01
F_v/F_m (maximal quantum yield)
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0.5
ab ab bc bc bc bc a ab ab ab c c
b)
It= 0.27 ± 0.06
α_{ETR} (photosynthetic efficiency)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a a b b a a a b c a a a
CCNS
CCNP+
LCNS
LCNP+
HCNS
HCNP+



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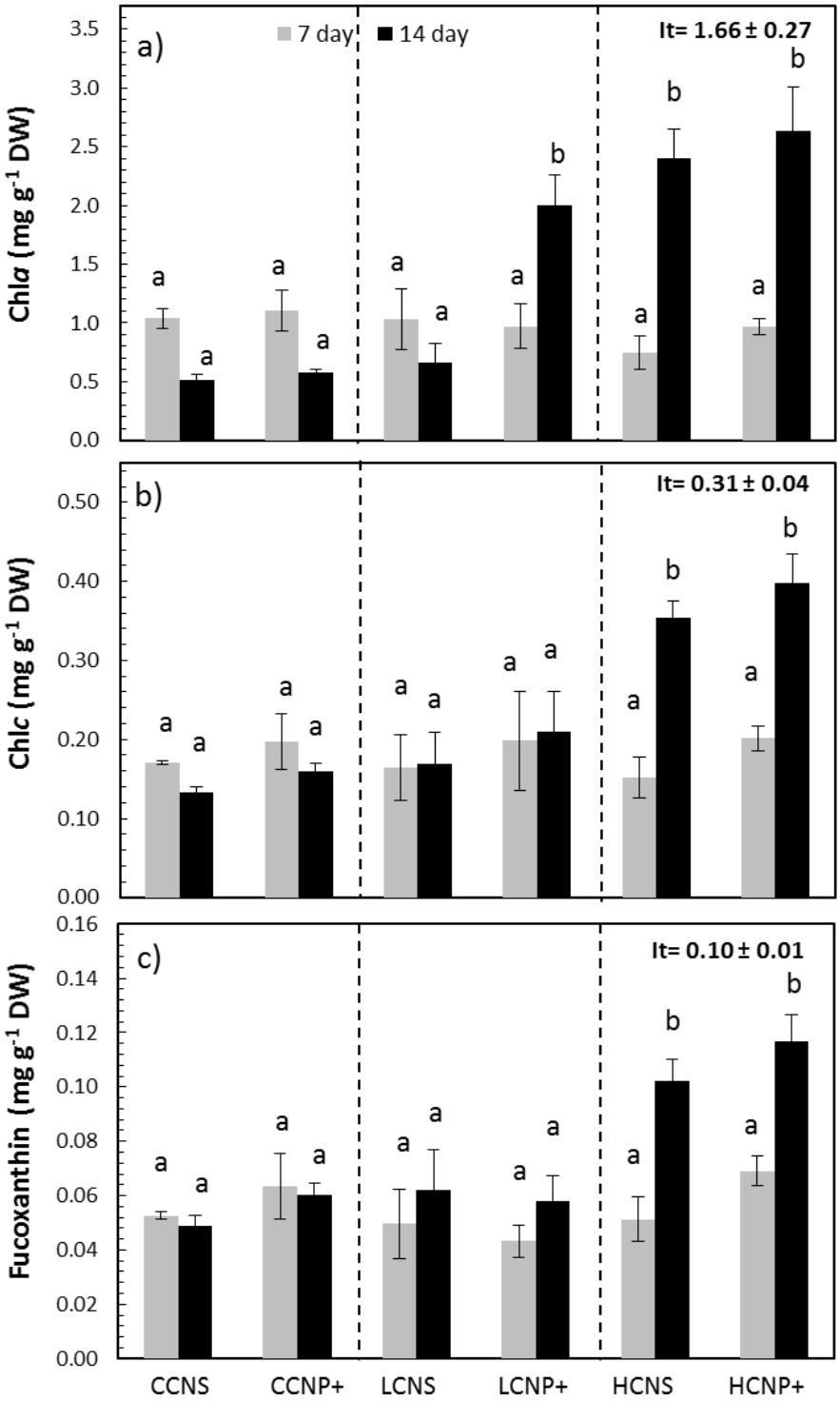

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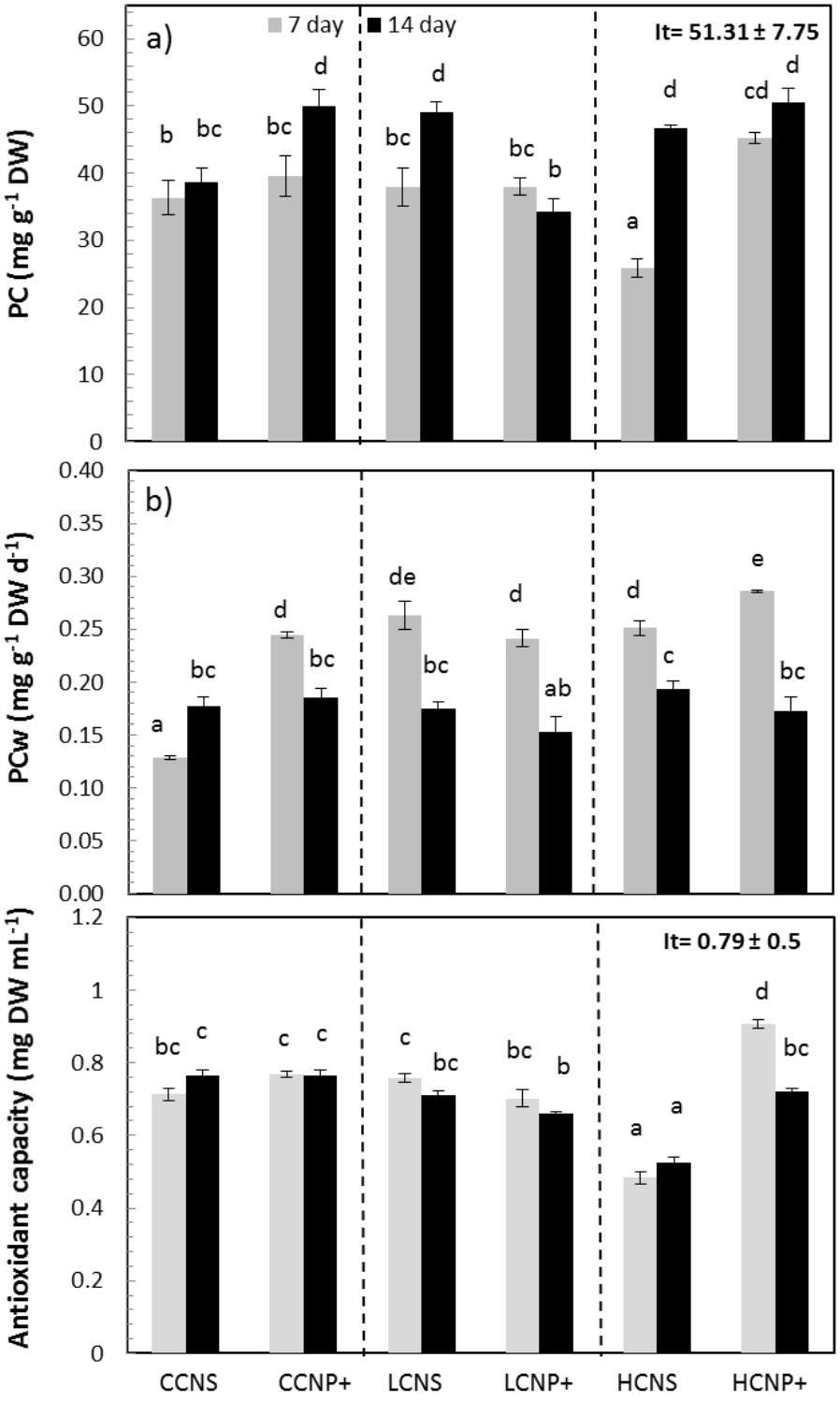

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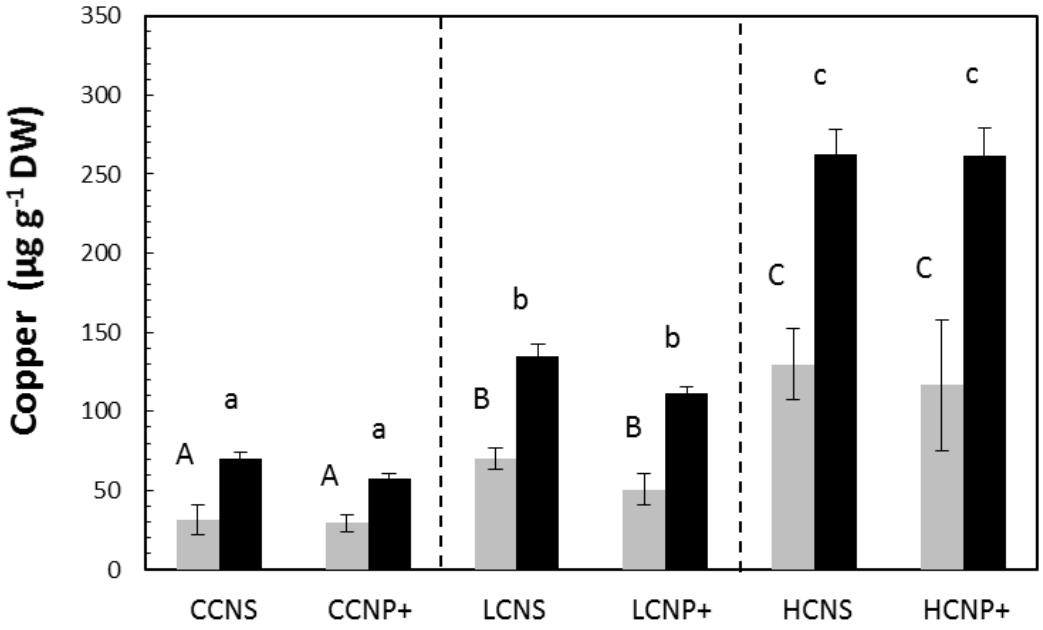
Copper (µg g-1 DW)
350
300
250
200
150
100
50
0
A
a
A
a
B
b
B
b
C
c
C
c
CCNS
CCNP+
LCNS
LCNP+
HCNS
HCNP+